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Journal of Biotechnology xxx (2005) xxx–xxx
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Functional large-scale production of a novel *Jonesia* sp. xyloglucanase by heterologous secretion from *Streptomyces lividans*

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Received 25 February 2005; received in revised form 13 July 2005; accepted 2 August 2005

Abstract

The gene encoding a novel xyloglucanase (Xeg) belonging to family 74 glycoside hydrolases was isolated from a *Jonesia* sp. strain through functional screening in *Escherichia coli*. The encoded xyloglucanase is a protein of 972 aminoacyl residues with a 23 residue aminoterminal signal peptide. Over-expression of Xeg in *B. subtilis* or *E. coli* failed. In contrast, Xeg was successfully over-expressed and secreted in *Streptomyces lividans* TK24. To this end Xeg was fused C-terminally to the secretory signal peptide of the subtilisin inhibitor protein (*vsi*) from *Streptomyces venezuelae*. The native Xeg signal peptide derived from *Jonesia* sp. is only poorly functional in *S. lividans*. Under optimal growth conditions, significant amounts of mature Xeg (100–150 mg/l) are secreted in the spent growth media. A protocol to rapidly purify Xeg to homogeneity from culture supernatants was developed. Biophysical and biochemical analyses indicate that the enzyme is intact, stable and fully functional. Xeg is the longest heterologous polypeptide shown to be secreted from *S. lividans*. This study further validates use of *S. lividans* for production of active heterologous proteins and demonstrates that heterologous polypeptides of up to 100 kDa are also tractable by this system.

Keywords: Xyloglucanase; Protein translocase; Signal peptide; Secretion; *Streptomyces lividans*; Protein secretion biotechnology

1. Introduction

Streptomyces lividans has been used for the heterologous secretion of several polypeptides of bacterial and eukaryotic origin for some recent examples

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0168-1656/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.jbiotec.2005.08.002

see (Hong et al., 2003; Lara et al., 2004; Ogino et al., 2004; Pozidis et al., 2001). In most cases, heterologous genes are fused to signal peptide sequences from highly expressed/secreted endogenous *Streptomyces* proteins (Lammertyn and Anné, 1998; Lammertyn et al., 1998). The resulting proteins are thus targeted to the *S. lividans* Sec translocase and very efficiently secreted directly into the growth medium. The absence of lipopolysaccharides, well-established genetic manipulation (Kieser et al., 2000), low protease activity and the avoidance of inclusion body formation renders *S. lividans* secretion a very attractive biotechnology platform.

Previously, we demonstrated that *S. lividans* can efficiently secrete active trimeric murine tumor necrosis factor alpha (mTNFα) into the growth medium (Lammertyn et al., 1997; Pozidis et al., 2001). In the course of this work, we used the transcription elements and signal peptide of the *Streptomyces venezuelae* CBS762.70 subtilisin inhibitor gene (Van Mellaert et al., 1998) (hereafter: *vsi*).

We now tested the *S. lividans* system for the production of a novel xyloglucanase (Xeg). Xyloglucan-specific endo-beta-1,4-glucanases (EC 3.2.1.151) are characterized by being active against xyloglucan, having no or limited activity on cellulose or cellulose derivatives like carboxymethylcellulose. The gene encoding this 972 aminoacyl-residue protein was isolated from a marine *Jonesia* sp. strain (Duffner and Sjøholm, 2003; WO patent application). Attemps to express Xeg in either *B. subtilis* or *Escherichia coli* were unsuccessful (data not shown). The mature region of Xeg (residues 24–972) is efficiently secreted from *S. lividans*. Isolation of the enzyme using a novel purification protocol revealed it to be catalytically functional and stable. These data extend use of the *S. lividans* secretion biotechnology platform to the production of high molecular weight polypeptides.

2. Materials and methods

2.1. Bacterial strains and recombinant DNA experiments

Growth and manipulation of *E. coli* and *Streptomyces* strains were as described (Ausubel et al., 1994; Kieser et al., 2000).

2.1.1. Isolation of Jonesia sp. DSM 14140

A small amount of marine sediment was distributed on minimal agar medium (pH 9) with 1% xyloglucan as carbon source and 0.025% AZCL-xyloglucan for xyloglucanase detection. After incubation (30 °C; 4 days) yellow colonies with xyloglucanase activity were selected. The strain was deposited at Deutsche Samlung für Mikroorganismen und Zellkulturen as DSM 14140. Partial 16S rDNA sequencing analysis was performed using the ARB package (Ludwig et al., 2004).

2.1.2. Construction of a Jonesia sp. expression library in E. coli

A Lambda ZAP library was prepared from the 3–10 kb fraction of Sau3A partially digested genomic DNA of *Jonesia* DSM14140 according to the manufacturer's instructions (Stratagene). Approximately, 10,000 plaque-forming units were plated on NZY-agar plates containing 0.1% AZCL-xyloglucan (MegaZyme, Ireland), using *E. coli* XL1-Blue MRF' (Stratagene), followed by incubation (37 °C; 24 h). Xyloglucanase-positive lambda clones were identified by the formation of blue hydrolysis halos around the positive phage clones. These were recovered from the screening plates by boring the TOP-agar slices containing the plaques of interest into 500 μl of SM buffer and 20 μl of chloroform and carrying out single-clone in vivo excision of the phagemids using *E. coli* XL1-Blue. Single colonies were re-streaked onto LB kanamycin agar plates containing 0.1% AZCL-xyloglucan. The xyloglucanase-positive clone was called *E. coli* XEG1020. Plasmid DNA was isolated from *E. coli* XEG1020 using Qiaspin plasmid preparation kit (Qiagen) and designated pXEG1020.

The nucleotide sequence of pXEG1020 was determined from both strands by the dideoxy chain-termination method (Sanger et al., 1977) using 500 ng of Qiaspin-purified template (Qiagen, USA), the Taq deoxy-terminal cycle sequencing kit (Perkin-Elmer, USA), fluorescently-labelled terminators and 5 pmol of either XEG1020 polylinker primers (Stratagene, USA) or custom synthetic oligonucleotide primers. (Genbank accession no. AX565635).

2.1.3. Cloning of Xeg in S. lividans

To over-express and secrete Xeg in *S. lividans* TK24, Xeg devoid of its predicted signal peptide (aa residues 24–972) was fused C-terminally to the sig-

nal peptide (VsiSP) of Vsi, the subtilisin inhibitor of *S. venezuelae* CBS762.70 (Van Mellaert et al., 1998). Two additional amino acids of the mature Vsi domain were maintained in the fusion protein VsiSP–Xeg. Its expression was placed under the control of the strong constitutive vsi promoter. To this end, the region of Xeg that encodes residues 24–972 was amplified by PCR using the primers XEG-mat (5′-TA**CTGCAG**CCACATCAACCCCCATGC) and XEG-stop (5′-AT**AAGCTT**CA TATGACTAACCTCTAGTC) and vector pXEG1020 as template. A *Pst*I restriction site (in bold) was included in the forward primer, while a *Hin*dIII site and a second in-frame stop codon (in bold and underlined, respectively) were introduced in the reverse primer. Upon cloning the PCR fragment in pGEM-T Easy (Promega), the resulting vector was first digested with *Pst*I, then treated with T4 polymerase in order to remove the 3′-protruding ends, and finally digested with *Hin*dIII. The obtained 2.77-kb fragment was subsequently cloned in *Dra*II/Klenow polymerase/*Hin*dIII-treated pBS-CBSS (Lammertyn et al., 1997).

A construct with Xeg carrying its native signal sequence was also made. The complete ORF was PCR-amplified using the primers XEG-full (5′-TA**CTGCAG**CCAAGGAGTGACGCGG, *Pst*I site in bold) and XEG-stop (see above), and pXEG1020 as template. Following a *PstI/Hin*dIII digest, the 2.85-kb PCR fragment was cloned into pBSvsi placing the full-length *xeg* gene under control of the *vsi* promoter.

In a next step, both expression/secretion cassettes were isolated as *Xba*I/*Hin*dIII restriction fragments and ligated in pIJ486 (Ward et al., 1986). Ligation mixtures were introduced in *S. lividans* by PEG-mediated protoplast transformation. Selection of xyloglucanase-producing transformants was carried out on MRYE medium (Anné et al., 1990) containing thiostrepton (5 μg/ml) and 0.05% AZCL-xyloglucan.

2.2. Bacterial growth and fermentation

Recombinant *Streptomyces* growth was in the presence of thiostrepton (5 μg/ml) to select for plasmid maintenance. Media used in this study were: phage medium (Korn et al., 1978) (per liter: 10 g glucose, 5 g tryptone, 5 g yeast extract, 5 g LabM, 0.74 g $CaCl_2{\cdot}2H_2O$, 0.5 g $MgCO_4{\cdot}7H_2O$, pH: 7.2), LB and TSB, a modification of the Trypticase Soy broth (Binnie et al., 1997) (per liter: 17 g tryptone, 3 g Bacto Soytone, 5 g NaCl, 2.5 g K_2HPO_4, pH: 7.2–7.5). Shake-flask studies were conducted in 2 l Erlenmeyer flasks containing 1 l liquid medium (at 180 rpm). Fermentation was carried out in a 20 l system (Bioengineering A.G., Switzerland).

2.3. Protein purification and chromatography

All chromatography resins and molecular weight markers were from Amersham. Purified proteins were stored at −20 °C. Molecular mass determination using SEC was as described (Pozidis, 2001 p. 26).

For Xeg purification from *S. lividans* culture supernatants cells were grown for 2 days in 500 ml phage medium and then transferred in 10 l TSB for 6 days.

Step 1 (Ultrafiltration). The 10 l supernatant was concentrated at 4 °C down to 0.05 l with a Minitan II System (Millipore; PTTK Filter Plates, 30.000 NMWL).

Step 2 (Ammonium sulfate fractionation). Polypeptides in concentrated spent media were precipitated by addition of $(NH_4)_2SO_4$ (45% saturation; 4 °C) and collected by centrifugation (Sorvall, JA20 rotor, 4 °C, 20 min, 10,000 rpm). $(NH_4)_2SO_4$ was added to the supernatant (65% saturation). Pellets harvested by centrifugation contain Xeg and were resuspended in 5 ml of buffer A (50 mM Tris–HCl pH 8.0, 0.15 M NaCl).

Step 3 (Size exclusion chromatography (SEC)). Samples from Step 2 (1 ml) were chromatographed on a Sephacryl S-200HR 26/40 column equilibrated with buffer A and 2 ml fractions were collected.

Step 4 (Ion exchange chromatography). Pooled SEC fractions from Step 3 containing Xeg were diluted with 50 mM Tris–HCl pH 8.0 (final NaCl concentration 0.02 M) and were loaded on a Mono Q HR 5/5 (equilibrated with buffer B: 50 mM Tris–HCl pH 8.0, 0.02 M NaCl). The column was washed with 10 column volumes of buffer B and proteins were eluted with a 120 ml 0.02–0.4 M NaCl linear gradient.

2.4. Intrinsic fluorescence–thermal stability

Tryptophan fluorescence emission of Xeg (0.25 μM; 50 mM Tris–HCl pH 7.2, 150 mM

NaCl, 5 mM β-mercaptoethanol) as a function of temperature (4–82 °C; 0.8 °C/min) was monitored at 345 nm (exc. 297 nm; data interval 0.5 min; slits were set at 2.5/20). Data were analyzed by non-linear regression as described (Vrontou et al., 2004).

2.5. Xyloglucanase functional assay

Xeg activity was measured by following the hydrolysis of AZCL-xyloglucan. 900 μl substrate (0.2% AZCL-xyloglucan in 0.2 M sodium phosphate buffer pH 6.0, 0.01%Triton-X-100) was preheated (30 °C; 10 min) and then 100 μl of enzyme in 0.01% Triton-X-100 was added. After incubation (30 °C; 30 min), the reaction was stopped by transferring the samples on ice. After centrifugation (1 min; 20,000 × *g*) the supernatant was transferred to a micro titer plate and was measured at 595 nm in an Assys Hitech spectrophotometer. Xeg1 xyloglucanase from *B. licheniformis* (Novozymes) was used as standard under the same reaction conditions.

2.6. Miscellaneous

Chemicals were from Sigma. Bacto Soytone from DIFCO Laboratories. DNA enzymes were from New England Biolabs and oligonucleotides from Eurogentec. Amino-terminal sequencing was performed at Alta Bioscience (UK).

3. Results

3.1. Molecular cloning of a Jonesia sp. xyloglucanase

Jonesia sp. strain DSM14140, was isolated from a marine mud sample collected on a tidal flat close to the Danish island Rømø. The strain was identified as a *Jonesia* sp. by partial 16S rRNA analysis having 98.6% sequence identity with *Jonesia quinghaiensis* (Schumann et al., 2004) from positions 10 to 506 of the 16S gene. To obtain sufficient amounts of the xyloglucanase a genomic DNA library was constructed and screened for endoglucanase activity using *E. coli* strain as a host (see Section 2). One clone that allowed *E. coli* to hydrolyse azurine-coupled (AZCL) xyloglucan on NZY plates was identified. Sequencing of the entire 8 kb insert of the excised plasmid revealed the presence of an open reading frame that encoded for a 972-residue long polypeptide (Fig. 1) homologous to xyloglucanases [xyloglucan-specific endo-beta-1,4-glucanase (EC 3.2.1.151)]. This protein is a novel

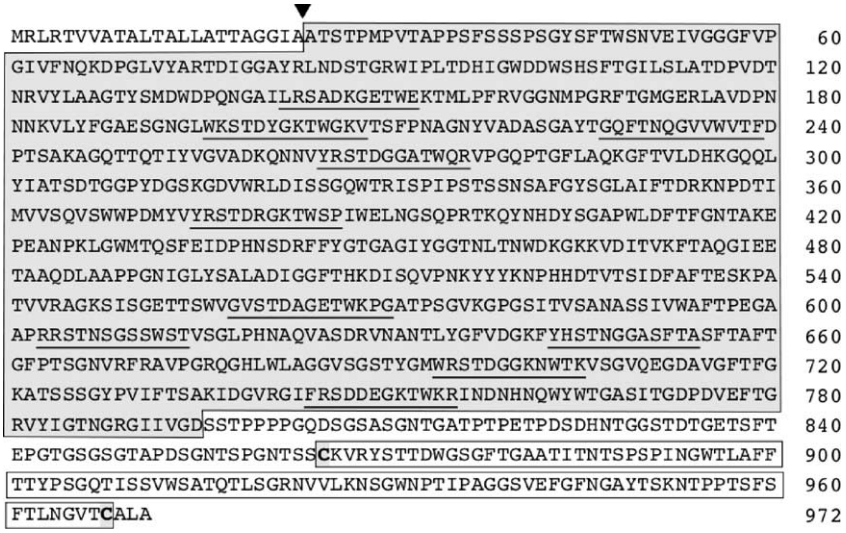


Fig. 1. Cloning of *Jonesia* sp. xyloglucanase gene. Predicted aminoacyl-residue sequence of *Jonesia* sp. Xeg. Greyed area represents the hydrolytic domain. BNR motifs are underlined. Filled arrow marks the site predicted by SignalP (v.3.0) (Bendtsen et al., 2004) to be a signal peptidase cleavage site. The two Cys residues that delineate the cellulose binding domain (CBD) are in bold and shaded.

Table 1
The closest homologues of *Jonesia* sp. Xeg

Database annotation	SwissProt code	Identity (%)	Hydrolytic domain	Cellulose binding domain	Organism
Putative secreted cellulase	O86727	47	BNR	Bacterial	*Streptomyces coelicolor*
Putative endo-1,4-beta-glucanase (celA3)	Q82M04	44	BNR	Bacterial	*Streptomyces avermitilis*
Beta-1,4-xyloglucan hydrolase (xghA)	Q70DK5	49	BNR		*Clostridium thermocellum*
Probably secreted sialidase; several ASP-boxes and dockerin domain	Q97KK0	46	BNR		*Clostridium acetobutylicum*
Endoglucanase C (eglC)	Q8TFP1	39	BNR	Fungal	*Aspergillus niger*
Avicelase III (aviII)	O74170	39	BNR	Fungal	*Aspergillus aculeatus*
CEL6	Q9P4T8	39	BNR	Fungal	*Agaricus bisporus*

Sequences were compared using Blast and aligned using Clustal W. BNR: Bacterial Neuraminidase Repeats (INTERPRO motif code/name: IPR002860/Glyco_hydro_BNR; PFAM motif code/name: PF02012/BNR; 10). Bacterial binding domain (INTERPRO motif code/name: IPR001919; Bac_celose-bind; PFAM motif code/name: PF00553/CBM_2; 1; SMART motif code/name: SM00637/CBD_II). Fungal cellulose binding domain (INTERPRO motif code/name: IPR000254/CBD_fungal; PFAM motif code/name: PF00734/CBM_1; SMART motif code/name: SM00236/fCBD).

xylo-endoglucanase (hereafter Xeg). The N-terminus of Xeg contains a potential signal peptide (residues 1–23) suggesting that it is secreted. Xeg is built of two main domains: (a) the hydrolytic domain (HD; residues 24–795) that contains several characteristic Bacterial Neuraminidase Repeats (BNR) motifs found in many glycosidases (Panel A, underlined) (Roggentin et al., 1989); (b) the C-terminal cellulose binding domain (CBD) delimited by Cys865 and Cys968. The CBD of Xeg is homologous to similar bacterial domains and a three-dimensional homology model can be derived from existing structures (data not shown). HD and CBD are joined by a linker containing several Pro, Gly, Ser and Thr residues. Sequence alignment places *Jonesia* sp. Xeg in xyloglucanase family 74. Close relatives include both bacterial and fungal enzymes (Table 1).

3.2. Functional expression of Xeg in S. lividans

To achieve high-level expression of Xeg we cloned *xeg* in appropriate vectors in *B. subtilis* and *E. coli*. However, several attempts to over-express Xeg in these systems failed (data not shown). To bypass this obstacle we used *S. lividans* TK24 as a secretion host. We successfully used this system previously for the production of tumor necrosis factor alpha (Pozidis et al., 2001). *S. lividans* TK24 cells were grown in three rich growth media using shake-flask cultures (Fig. 2): Luria-Bertani broth (LB; lane 2), phage medium (Korn et al., 1978) (lane 3) and a modified Trypticase Soy broth medium (TSB; lane 4) (Binnie et al., 1997). Supernatants were harvested after 5 days and secreted peptides analyzed by SDS-PAGE and Coomassie blue staining. Significant variation was observed in the overall protein profile of the spent media. Cells grown in LB (lane 2) and phage (lane 3) media showed little or

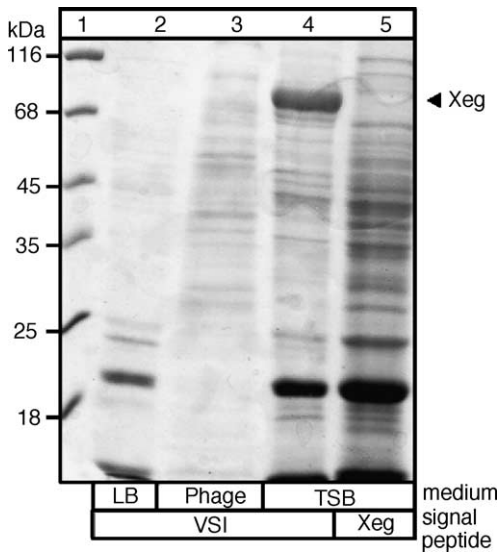


Fig. 2. Heterologous expression/secretion of Xeg. Xeg expression and secretion in *S. lividans*. Polypeptides (20 μg/lane) from culture supernatants (0.14 ml) from cells grown for 4 days in the indicated media (see Section 2) were harvested by precipitation with TCA (15%) analyzed by SDS-PAGE and stained with Coomassie blue. Lane 1, molecular weight markers: β-galactosidase (116 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease *Bsp98I* (25 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa). LB, Luria-Bertani broth; TSB, SoybeanTryptic broth.

no secretion of Xeg, while significant levels of secreted Xeg were detectable in the TSB spent medium (lane 4). Recombinant Xeg migrated with an apparent molecular weight of ~90–95,000 Da in close agreement with the predicted size (97 kDa) and represented ~50% of extracellular protein. Use of the native Xeg signal peptide from *Jonesia* sp. led to poor or no detectable Xeg secretion (lane 5). Therefore, only the VsiSP–Xeg construct was subsequently used.

3.3. Optimal conditions for Xeg secretion in S. lividans

To determine optimal culture conditions growth and secretion time-course experiments were performed. In TSB medium, the optimal culture time was found to be ~120 h (Fig. 3A) and the observed yield of secreted Xeg was 100–150 mg/l in different experiments. In this period of time, the pH of the culture increased from the initial value of 7.5 to a final value of 9.5 (lane 5) after 6 days of growth. Enhanced Xeg secretion correlated well with detection in the culture supernatant of xyloglucanase activity that is completely absent from TK24 (Panel B). Contrary to previous observations (Kim et al., 1998; Parro and Mellado, 1994; Pozidis et al., 2001) final Xeg secretion yield was not increased by addition of additional carbon source. In fact addition of dextrose at 3 g/l (lanes 6–9) or 6 g/l (lanes 10–13) reduced the amount of secreted Xeg (filled arrow) and other proteins (open stars; compare lanes 3–5 to 11–13), while secretion of other proteins remained unaffected or was enhanced (Fig. 3A, filled star; compare lanes 3–5 to 11–13). The observed Xeg secretion lag correlated with the lower pH measured in the medium under the same conditions.

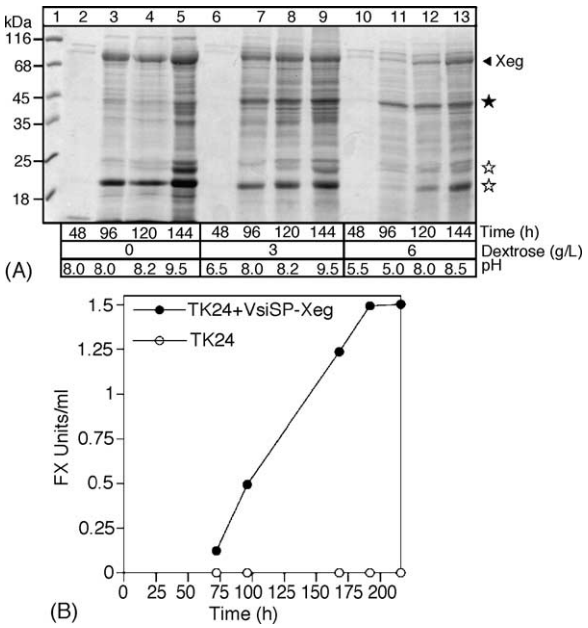


Fig. 3. Time course of Xeg secretion and the effect of additional carbon source. (A) Polypeptides from culture supernatants from cells grown for 4 days were analyzed by SDS-PAGE and stained with Coomassie blue. Lane 1, MW markers as in Fig. 2. Secreted Xeg (filled arrow) is indicated. Determined pH values of the cell-free spent media are indicated. Stars, abundant endogenous *S. lividans* secreted polypeptides. (B) *S. lividans* strain TK24 with or without pIJ486 expressing VsiSP–Xeg were grown as in (A). Polypeptides were isolated at the indicated time points and xyloglucanase activity determined (see Section 2).

3.4. Purification of Xeg secreted from S. lividans

We next proceeded to develop a simple and rapid four-step purification scheme (Fig. 4; Table 2; see Section 2) for Xeg present in the spent culture medium of *S. lividans* (lane 2). This involved concentration by ultrafiltration (lane 3), ammonium sulfate precipitation (lane 4), size-exclusion chromatography (lane 5) and ion-exchange chromatography (lane 6). Xeg was thus purified at more than 98% purity as judged by Coomassie-stained SDS-PAGE gels. The determined

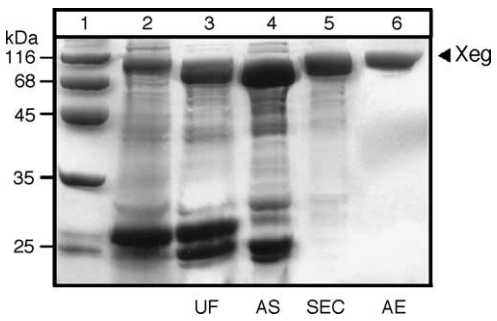


Fig. 4. Large-scale purification of *Jonesia* sp. Xeg expressed and secreted in *S. lividans* culture supernatants. Protein samples (15–30 μg/lane) from the various purification steps (see Table 2 and Section 2) were analyzed by SDS-PAGE and were stained with Coomassie blue. Lane 1: MW markers as in Fig. 2. UF, ultrafiltration retentate; AS, material percipated with $(NH_4)_2SO_4$ at a 45–65% saturation cut; SEC, size-exclusion chromatography; AE, anion-exchange chromatography.

Table 2
Purification table of *Jonesia* sp. xyloglucanase expressed as a secretory protein from *S. lividans* TK24

Step	Volume (ml)	A_{vol} (U/ml)	C_{prot} (mg/ml)	A_{tot} (U)	P_{tot} (mg)	A_{spec} (U/mg)	Purification factor	Yield (%)
Crude extract	10000	0.48	0.07	4800	700	6.85	1	100
Ultrafiltration	100	39	3.2	3900	320	12.2	2	82
Na_2SO_4 (65% sat)	6	490	40	2940	240	12.2	2	61.2
SEC (S-200)	100	27.7	0.97	2770	97	28.5	4	57.7
Anion exchange (MonoQ)	3.5	340	11.2	1190	39.2	30.3	4	24.8

Where A_{vol}: volume activity (U/ml); C_{prot}: protein concentration (mg/ml); A_{tot}: total activity = $V \times A_{vol}$; P_{tot}: total protein mass = $V \times C_{prot}$; A_{spec}: specific activity = A_{vol}/C_{prot}; purification factor: A_{spec} step X/A_{spec} Cex; yield: (A_{tot} step X/A_{tot} Cex) × 100.

N-terminal residues of the purified protein (Glu-Ala-Ala-Thr-Ser) are those predicted from the sequence. Therefore, the vsi signal peptide is correctly processed from Xeg secreted from *S. lividans* and the generated mature N-terminus remained intact.

3.5. Physical and biochemical characterization of secreted recombinant Xeg

To evaluate the quality of secreted Xeg produced by *S. lividans* we determined whether it was structurally and functionally intact. Analytical size exclusion chromatography revealed that Xeg has a native molecular weight of ∼95 kDa and is therefore monomeric. The purified protein is monodisperse without any visible signs of aggregation. Recombinant Xeg is a stable enzyme with an apparent T_m of 53.2 °C as revealed by monitoring changes in intrinsic fluorescence during thermal denaturation (Fig. 5).

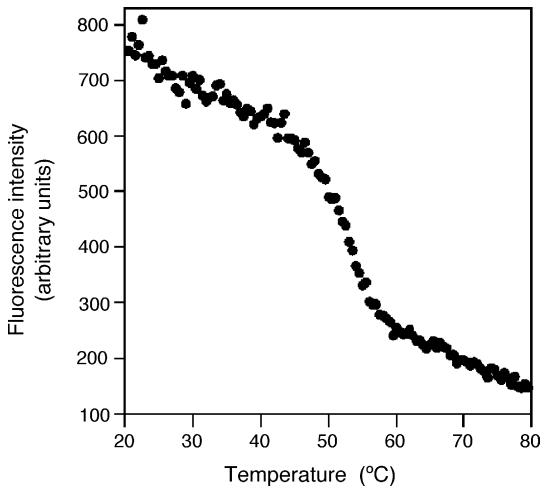


Fig. 5. Physical and functional characterization of *S. lividans*-secreted Xeg. Thermal denaturation curves. Purified Xeg (35 μg) was exposed to gradual temperature rise and changes in intrinsic fluorescence were monitored as described (Vrontou et al., 2004). Xeg has 34 Trp residues.

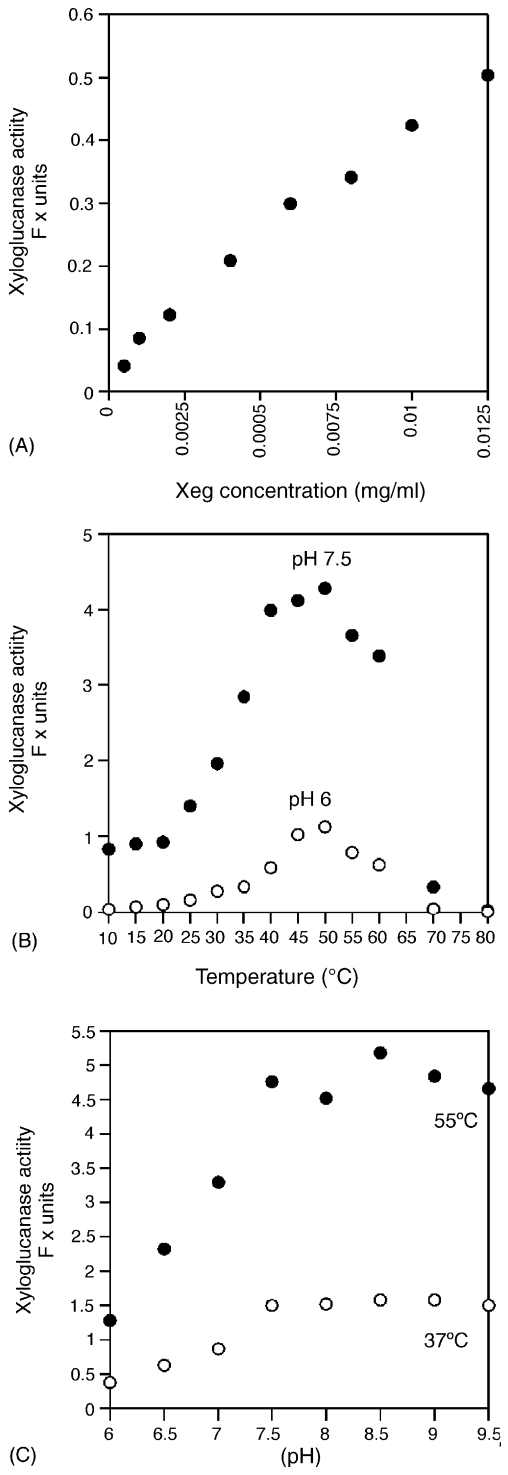
We next examined the catalytic activity of recombinant Xeg. The enzyme is highly active in hydrolyzing AZCL-xyloglucan in a linear fashion as a function of concentration (Fig. 6A). Hydrolysis is optimal at 50–55 °C (Fig. 6B and C) and at a pH of 7.5–9 (Fig. 6B and C).

We conclude that Xeg produced as a secreted polypeptide from *S. lividans* cells is structurally intact and biologically fully functional.

4. Discussion

We present a pilot scheme for large-scale production and secretion of functional xyloglucanase from the *Jonesia* sp. by *S. lividans*. Use of this expression-secretion system was prompted by failure to establish high-level expression of the enzyme using the traditional bacterial hosts *B. subtilis* or *E. coli*. This observation reinforces the need for alternative host strategies for protein production and strengthens use of *S. lividans*-based protein secretion biotechnology.

Xeg can be produced at high levels from *S. lividans* under specific fermentation regimes where the secreted form represents ∼50% of the total protein present in the spent growth media (Figs. 2 and 3). The evolutionary proximity between the two organisms (both members of the *Actinomycetales*) may have facilitated high level expression of the *Jonesia* sp. gene in *S. lividans*. A native *S. lividans* signal peptide is required for secretion and cannot be replaced by the *Jonesia* signal peptide. This reconfirms the importance of appropriate signal peptides for optimal heterologous



secretion (Lammertyn and Anné, 1998). A secretion yield of 100–150 mg/L seen with small-scale cultures is among the best obtained to date from this host organism. Significant amounts of the protein are observed after 4 days of culture and accumulated protein was observed after prolonged incubations suggesting that secreted Xeg is proteolyticaly stable and remains unaffected by *S. lividans* secreted proteases. This was also observed for many heterologous proteins secreted by *Streptomyces* (Lara et al., 2004; Pimienta et al., 2002; Pozidis et al., 2001) but not for others (DelaCruz et al., 1992). Addition of optimal carbohydrate amounts can lead to maximal product yields at reduced culture times (Pozidis et al., 2001). However, this was not the case with Xeg secretion (Fig. 3). In fact an increase of carbon content in the medium causes reduction of Xeg synthesis and/or secretion (Fig. 3, lanes), while it leaves expression/secretion of other polypeptides unaffected (Fig. 3). This and other studies (Fernandez-Abalos et al., 1997; Harchand and Singh, 1994; Kim et al., 1998) indicate that growth conditions exert a degree of regulation of the secretory protein genes and/or the secretion pathway genes not seen in the *E. coli* system. To further develop *Streptomyces* secretion biotechnology it is important to understand how different fermentation regimes affect protein secretion of native and heterologous proteins. The varying profiles of endogenous *Streptomyces*-secreted proteins under different growth conditions (Figs. 2 and 3), makes the establishment of strict culture conditions very important for reproducibility of subsequent purification schemes. Also fermentor growth of the cells results in reduced final Xeg yield (Table 2) and will need to be optimized. Finally, the production of highly expressed endogenous *S. lividans* proteins (e.g. Fig. 3, filled and open stars) could compete with secretion of the heterologous protein of interest and should be controlled.

Fig. 6. Biochemical activity of of *S. lividans*-secreted Xeg. Xyloglucanase activity by Xeg (20 μg/ml; B and C) determined by hydrolysis of AZCL-xyloglucan (see Section 2). (A) Concentration-dependence of the xyloglucanase activity. (B) Temperature-dependence of Xeg xyloglucanase activity at the indicated pH values. pH 7.5 was maintained using 100 mM Tris–Cl and pH 6 was maintained using 0.2 M Na-phosphate buffer. (C) pH-dependence of Xeg xyloglucanase activity. Buffering at the indicated values was established using (100 mM bis–tris–propane–Cl).

Xeg secreted by *S. lividans* is a stable polypeptide (Fig. 5) and is highly active in biochemical assays (Fig. 6). Xyloglucanases are important industrial enzymes with potential application both as detergent additive, in the textile industry and for improving the economics of biomass conversion. The successful expression of this novel xyloglucanse as a secretory protein from *S. lividans* described here is compatible with many of the established processes of industrial enzyme biotechnology and opens new areas for industrial application of xyloglucanases.

Elongated polypeptides can challenge heterologous expression. The ∼100 kDa Xeg is the longest heterologous polypeptide that has been successfully secreted to date from *S. lividans*. A particular capacity to secrete large proteins may represent a particular adaptation of the streptomycetes Sec pathway, since the close relative *S. coelicolor* is predicted to secrete more than 20 proteins that are longer than 1000 residues, including a 2314 aa putative phosphatase (SCO6428). By comparison only four *E. coli* secreted/outer membrane proteins longer than 1000 aa are known. These observations and the excellent Xeg secretion yield suggest that the *S. lividans* cell factory may be well suited for large-scale production of other elongated polypeptides of biopharmaceutical or industrial importance in an active form.

Acknowledgements

We would like to acknowledge the technical assistance of Githa Hatt and Charlotte Østbo. This study was supported by the European Union (grant QLK3-CT-2002-02056 to A.E., J.A. and T.S.), the Greek Secretariat of Research and Technology (grant 03AKMON46 to A.E.) and by the FWO (Fund for Scientific Research – Flanders; grant G.0389.05 to J.A.). K.V. is a research fellow of IWT (Institute for the Promotion of Innovation by Science and Technology in Flanders). The initial isolation and cloning of Xeg was supported by grant MAS3-CT97-0125 (to C.S. as partner in the Margenes project) from the European Union.

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